A sustainable use of Ricotta Cheese Whey for microbial biodiesel production

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Abstract

The increasing demand of plant oils for biodiesel production has highlighted the need for alternative strategies based either on non-food crops or agro-industrial wastes that do not compete with food and feed production. In this context, the combined use of wastewater and oleaginous microorganisms could be a valuable production option. Ricotta cheese whey (RCW), one of the major byproducts of the dairy industry, is produced in very high and steadily increasing amounts and, due to its high organic load, its disposal is cost-prohibitive.

In the present study, in order to assess the adequacy of RCW as a growth medium for lipid production, 18 strains of oleaginous yeasts were investigated in shaken flask for their growth and lipid-producing capabilities on this substrate. Among them, *Cryptococcus curvatus* NRRL Y-1511 and *Cryptococcus laurentii* UCD 68-201 adequately grew therein producing substantial amounts of lipids (6.8 and 5.1 g L^{-1}, respectively). A high similarity between the percent fatty acid methyl esters (FAME) composition of lipids from the former and the latter strain was found with a predominance of oleic acid (52.8 vs. 48.7%) and of total saturated fatty acids (37.9 vs. 40.8%). The subsequent scale transfer of the *C. laurentii* UCD 68-201 lipid production process on RCW to a 3-L STR led to significantly improved biomass and lipid productions (14.4 and 9.9 g L^{-1}, respectively). Although the *C. laurentii* FAME profile was modified upon process transfer, it resembled that of the Jatropha oil, a well established feedstock for biodiesel production. In conclusion, *C. laurentii* UCD 68-201, for which there is very limited amount of available information, turned out to be a very promising candidate for biodiesel production and wide margins of process improvement might be envisaged.

Keywords:

Ricotta Cheese Whey, *Cryptococcus laurentii*, oleaginous yeasts, biodiesel, single cell oil

1. Introduction

Ricotta cheese whey (RCW) is a by-product of dairy industry derived from ricotta cheese production. During this process, the whey is heated at 80-90 °C and generally added with organic acids and salts to induce the denaturation and consequent precipitation of whey proteins. The curd thus obtained is allowed to cool and filtered to separate the solid part (ricotta) from the liquid waste which is referred to as RCW (Lavarda, 1972).

Although the large majority of RCW is produced in the Mediterranean basin (about 1 Mton per year only in Italy) (Sansonetti et al., 2009), the manufacturing of ricotta cheese is also widespread in USA, where this product is often referred to as ricottone. Therefore, the technical hurdles related to either upgrading or disposal of this dairy byproduct are not only a regional issue. The typical composition of RCW is 4.8-5.0% lactose, 1.0-1.3% salts, 0.15-0.22% proteins, 0.20-0.25% organic acids and 0.20% fats with a COD ranging between 50.000 to 80.000 mg L^{-1} (Sansonetti et al., 2009). However, despite the presence of residual nutrients, the aforementioned presence of additives impairs its nutritional value and, consequently, its profitable use as a feed for livestock, causing additional costs for the dairy industry due to the need of its disposal. Moreover, RCW might cause a considerable environmental impact in case of an inappropriate disposal procedure.

Despite the appreciable sugar content in RCW which makes it a putative candidate as a growth medium in microbial production processes, only few studies have been conducted for this purpose. These studies include bio-ethanol production by *Kluyveromyces marxianus* (Sansonetti et al., 2009; Zoppellari and Bardi, 2013) and lactic acid production by mixed and pure bacterial cultures (Secchi et al., 2012). Most of research has focused instead on cheese whey, which, however, differs substantially from RCW, due to its lower concentration of salts and organic acids and higher content of whey proteins (Ykema et al., 1988; Takakuwa and Saito, 2010).

Microbial biodiesel production is among the most promising upgrading options of low cost feedstocks characterized by a high content in carbohydrates associated with low nitrogen content. In this respect, RCW seems to meet these nutritional requirements. Biodiesel is a mixture of fatty acids alkyl monoesters derived from renewable resources such as higher plant oils, or, to a lesser extent, animal fats and waste cooking oil (Srivastava and Prasad, 2000) produced from transesterification reactions of triacylglycerides (TAG). Compared to conventional diesel, it has several advantages, being non-toxic, renewable and biodegradable. However, the rising costs of plant oils, from which the large majority of biodiesel is derived (around 95% of total world production), and the increasing demand for biofuels have given rise to concerns about land-use practices, increase of food price and oil production strategies (Atabani et al., 2012). Therefore, the use of microbial oils might represent a promising alternative to mitigate the problems associated with the ‘‘food vs. fuel’’ issue. Several microorganisms, belonging to yeasts, molds and microalgae, have the reported ability to accumulate intracellular lipids up to 70% (w/w) of their dry weight; the microbial fatty acid composition is often similar to that of vegetable oils used for biodiesel (Cristophe et al., 2012). Among them, oleaginous yeasts (OY) seem to be more suitable for an industrial production, being easy to cultivate, fast-growing and receptive to scale-up (Li et al., 2008).

Lipid accumulation in OY occurs through two different mechanisms depending on the nature of growth medium. “Ex-novo” synthesis is observed on hydrophobic substrates, while “de novo” synthesis occurs on sugar-based media in concomitance with a shortage of a key element, usually nitrogen.

In this study, the adequacy of RCW as a growth medium for the production of microbial oil was assessed. In fact, apparently the chemical composition of RCW characterized by high C/N ratios might be compatible with microbial “de novo synthesis” of lipids (Pirozzi et al., 2013). The exploitation of RCW in this direction would be in line with sustainability since it would enable a

partial replacement of edible oils as feedstock for biodiesel, giving back land use to food crops. Furthermore, in view of a possible development of a circular economy (Cicatiello et al., 2016), RCW upgrading would be beneficial for both dairy industries, due to a reduction of production costs, and for the environment since the spent medium derived from fermentation, would have a negligible organic load.

To this aim, a screening was initially performed with several OY belonging to well known lipid-producing species, some of which previously isolated from dairy products (Corbo et al., 2001), to assess their respective abilities to grow and produce lipids on that dairy byproduct and to determine productivities. The fatty acid methyl ester compositions derived from transesterification of lipids produced by some selected strains were analyzed and compared with well established feedstocks for biodiesel production. The most promising strain was then tested in a stirred tank reactor in view of a preliminary assessment of the process scale transfer feasibility.

2. Materials and methods

2.1 Microbial strains and maintenance

The strains under study were obtained from various culture collections or were isolated from environmental matrices and were chosen on the basis of their reported lipid accumulation ability on synthetic media. *Candida rugosa* NRRL Y-95, *Cryptococcus curvatus* NRRL Y-1511, *Lipomyces starkeii* NRRL 11557, *Rhodosporidium torouloides* NRRL Y-1091, *Rhodosporidium torouloides* NRRL Y-17902, *Trichosporon fermentans* NRRL Y-1492, *Yarrowia lipolytica* NRRL YB-423, *Y. lipolytica* NRRL Y-1095 and *Y. lipolytica* NRRL Y-7208 were provided by the ARS Culture Collection (NRRL, Peoria, IL). *Cryptococcus albidus* UCD 68-150, *C. albidus* UCD 68-174, *Cryptococcus laurentii* UCD 68-201, *Rhodotorula glutinis* UCD 68-255 and *Rhodotorula minuta* UCD 68-280 were obtained from the UCD Collection (Davis, California), while *Rhodotorula glutinis* DBVPG 3853 from DBVPG Collection (Perugia, Italy). *Pichia*

guilliermondii 1067 and *Pichia anomala* AN/4 were a kind gift of Prof. Cardinali (University of Perugia; Italy). *Pichia membranifaciens* 6C1 was isolated from olive brine (Crognale et al., 2012) and identified on the basis of its ITS sequence (GenBank Accession number JN900498).

During the study, the strains were maintained on potato dextrose agar (PDA) slants at 4°C and sub-cultured every month.

2.2 Growth medium

RCW was collected from a cheese processing plant (Formaggi Boccea S.r.l., Rome, Italy) and stored at -20 °C until used. RCW had the following characteristics (g L^{-1}): dry weight, 48.2±4.10; Chemical Oxygen Demand (COD), 43.5±3.8; Total Organic Carbon (TOC), 16.3±1.4; lactose, 40.2±0.8; galactose, 1.6±0.2, total nitrogen, 0.053±0.04; protein, 0.008±0.001; C/N, 307; ash, 4.5±0.4. Initial pH was 5.8. After thawing, the RCW was centrifuged (8000 x g, 15 min), two-fold diluted with deionized water, added with $(NH_4)_2SO_4$ so as to reach a C/N ratio of 55 and finally its pH was adjusted to 5.5 with 0.1 M NaOH.

2.3 Culture conditions

2.3.1 Shaken flask experiments

The microorganisms mentioned above were firstly screened in shaken flasks to select the best strain in terms of biomass and lipid production. Regardless of the strain, each inoculum was obtained by suspending 72-h-old PDA slants with sterile physiological solution. Inocula were added to 250-mL Erlenmeyer flasks containing 50 mL of RCW-based medium so as to yield an initial value of optical density of 600 (OD_{600}) equal to 0.2. After inoculation, flasks were incubated at 30 °C in an orbital shaker (185 rpm) for 5 days. Samples were collected on a daily basis. All experiments were performed in triplicate. Biomass ($Y_{X/S}$) and product ($Y_{P/S}$) yields were determined by relating yeast biomass and lipid production, respectively, to the extent of total

147 sugars consumed at the time of maximal lipid concentration. Specific product yield ($Y_{P/X}$) was
148 calculated by relating lipid to biomass production.
149
150 *2.3.2 Bioreactor experiments*
151 Bioreactor experiments were performed in a 3-L jacketed bench-top stirred tank reactor (STR)
152 (Applikon, Schiedam, NL) filled with 2 L of medium. The bioreactor was endowed with a top
153 stirrer bearing two six-blade Rushton-type turbines (diameter 4.5 cm, blade width 1.4 cm, blade
154 length 1.4 cm) and three baffles (width 1.4 cm). Air was introduced through a perforated pipe
155 sparger located under the bottom turbine. The top plate was equipped with the following probes:
156 dissolved oxygen and pH sensors (Applikon) and a PT 100 temperature sensor. Standard
157 bioprocess conditions were as follows: impeller speed, 600 rpm (impeller tip speed = 141 cm s^{-1});
158 aeration rate, 1.5 vvm; temperature, 30 °C; initial dissolved oxygen concentration, 100% of
159 saturation. Silicon Antifoam 289 (0.5 mL L^{-1}) (Sigma Chemical Co., St Louis, MO, USA) was
160 added before inoculation and an additional 1 mL L^{-1} was added when needed. The fermentation
161 parameters (temperature, pH and dissolved oxygen) were monitored in bioreactors by an ADI 1030
162 (Applikon) adaptive/PID digital controller.
163 Pre-inoculum was grown at 30 °C in shaken flasks on Potato Dextrose Broth medium for 24 h,
164 under orbital shaking (185 rpm) and added to the reactor so as to yield an initial value of OD_{600}
165 equal to 0.2. Reactor experiments were performed in duplicate and culture samples collected every
166 12 h. Besides yields ($Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$), kinetic parameters, such as specific growth rate (μ), lipid
167 and biomass production rates (r_P and r_X, respectively) and N and total sugars consumption rates (r_N
168 and r_S, respectively) were calculated, as described in subsection 2.4.
169
170 *2.4 Determination of yields and rates*
171 The specific growth rate (μ) was calculated according to the following equation

$$\mu = \frac{1}{X} \cdot \frac{\delta X}{\delta t} \quad (1)$$

where X is the biomass concentration (g L^{-1}) at time t (h).

The biomass yield ($Y_{X/S}$) and product yield ($Y_{P/S}$) were calculated according to Equations (2) and (3), respectively:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (2)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (3)$$

where ΔX and ΔP are the amounts of biomass and product (either lipids or biodiesel), respectively, and ΔS is the amount of substrate consumed.

The volumetric growth and production rates (r_X and r_P, respectively) were calculated according to Equations (4) and (5) by relating the amounts of biomass and biodiesel, respectively, to the time required to attain the lipid production peak (Δt):

$$r_X = \frac{\Delta X}{\Delta t} \quad (4)$$

$$r_P = \frac{\Delta P}{\Delta t} \quad (5)$$

substrate (r_S) and nitrogen (r_N) consumption rates were calculated by Equation (6) by relating the amounts of total sugars and nitrogen consumed, respectively, to the time required to attain the lipid production peak (Δt):

$$r_{[S,N]} = \frac{\Delta [S, N]}{\Delta t} \quad (6)$$

190 *2.5 Analytical methods*

191 Cell biomass was collected from 5 mL-samples in pre-weighed Falcon tubes. The suspension was
192 centrifuged at 8000 x g for 10 minutes and washed 3 times with distilled water. Dry cell weight
193 was determined gravimetrically after lyophilisation for 48 h.

194 Total sugars content was determined by using the phenol-sulphuric acid method (Dubois et al.,
195 1956). The concentrations of lactose and galactose were determined by ion-moderated partitioning
196 chromatography in a Varian HPLC system equipped with an Aminex HPX 87-P column (Biorad
197 Laboratories, Milan, IT). Samples were eluted with Milli-Q water (flow rate 0.6 mL min^{-1}) at 65
198 °C and the elution profile was monitored by an IR4 refractive index detector (Varian, Sunnyvale,
199 CA). Determination of lipid content was performed according to the method of Izard and
200 Limberger (2003). COD and TOC were determined according to Standard Methods (APHA,
201 2005). Ashes were determined gravimetrically after 12-h ignition at 550 °C in a muffle furnace.

202 Nitrogen was determined by a modified Kjeldahl method (Domini et al., 2009). Digestions were
203 carried out in a batch microwave digestion system (MarsXpress, CEM, Matthews, NC, USA) at
204 500 W power, 200 °C for 10 minutes by adding a mixture of 37% HCl (Carlo Erba Reagenti,
205 Milan, Italy) and 30% H_2O_2 (Merck KGaA, Darmstadt, Germany) to 1 mL sample. Afterwards,
206 nitrogen present in the form of ammonium was determined spectrophotometrically at 650 nm using
207 the nitroprusside method described by Anderson and Ingram (1993).

208 Yeast cells were stained with Sudan Black B followed by counterstaining with Safranin to detect
209 the presence of intracellular storage lipids as described by Ravikumar and collaborators (2012).
210 Characterisation of lipid profiles was performed by a direct transesterification on lyophilised cells
211 (Schutter and Dick, 2000) to obtain fatty acid methyl esters (FAMEs), which were analysed by a
212 Master GC gas chromatograph (DANI Instrument SpA, Cologno Monzese, Italy) equipped with a
213 Rxi-5MS (Restek, Germany) capillary column (0.25 mm id x 30 m length). FAMEs were eluted by
214 using the following program: isothermal at 89 °C for 2 min; temperature gradient from 89 to 280

°C at a 6 °C min^{-1}; hold at 280 °C for 5 min. The temperatures of the injector and flame ionization detector were set at 280 and 300 °C, respectively. Each FAME was identified by comparing its retention time with that of authentic standards contained in the FAME Mix C8-C24 (Sigma Aldrich, 18918-1AMP, USA). For quantification purposes, an internal standard (i.e., methyl nonadecanoate) was added to each sample prior to the transesterification. Biodiesel yield was calculated by relating the amounts of FAME to those of cellular lipids in dry biomass.

3. Results

3.1 Screening of yeast strains

Table 1 comparatively reports biomass and lipid productions and related yields calculated at the time (t) of maximal lipid accumulation. Regardless of the strain under study, the duration of the production process was brief with best lipid accumulation being observed between 48 and 96 h.

Among the tested strains, *C. curvatus* NRRL Y-1511 and *C. laurentii* UCD 68-201 proved to efficiently grow on RCW-based medium, achieving biomass productions of 10.77 and 7.28 g L^{-1}, respectively. The ability of the former and the latter strain to use RCW as a growth substrate was confirmed by marked reduction in COD (86.7 and 77.9%, respectively) (data not shown). *C. curvatus* was able to produce 6.83 g L^{-1} of intracellular lipids which amounted to 63% of biomass dry weight, while *C. laurentii* achieved a slightly lower production level (5.06 g L^{-1}) but with a higher yield with respect to biomass, amounting to 70%.

The other strains showed greater difficulties in adapting to this substrate but some of them, despite a low growth, were able to accumulate high percentages of lipids with respect to biomass. In particular, $Y_{P/X}$ values in *L. starkeii* and *R. toruloides* NRRL Y-17902 amounted to 0.63 and 0.79 although their biomass productions were 0.79 and 0.64 g L^{-1}, respectively (Table 1). Conversely, although *R. glutinis* UCD 68-255, *T. fermentans* and *P. membranifaciens* exhibited substantial

growth in the RCW-based medium their relative $Y_{P/X}$ values were very low (i.e., 0.09, 0.20 and 0.13, respectively).

Thus, on the basis of the performance observed in shaken cultures, the determination of the lipid profiles was limited to some selected strains. Figure 1A, reporting the percent concentrations of each identified fatty acid with respect to total FAME, shows that in all the strains considered, the main fatty acids were palmitic (C16:0), oleic (C18:1 Δ9), linoleic (C18:2 Δ9,12) and stearic (C18:0) acids, while the concentrations of polyunsaturated fatty acids (PUFAs), such as linolenic (C 18:3 Δ 9,12,15), eicosadienoic (C20:2 Δ11,14) and arachidonic (20:4 Δ5,8,11,14) acids, were either negligible or even absent. The FAME profiles of *C. curvatus*, *C. laurentii* and *L. starkeii* were rather similar while that of *R. toruloides* greatly differed from the others due to its higher content in total saturated (52.6%) and polyunsaturated (15%) fatty acids, as shown in Figure 1A.

Although the biodiesel yields of *C. curvatus* and *C. laurentii* (35.77±3.51% and 27.1±4.80%, respectively) were lower than those of *R. toruloides* and *L. starkeii* (88.90±11.2% and 54.32±3.99%, respectively) (Figure 1B), their volumetric lipid productions, lipid accumulation capacities and specific fatty acid profiles, were deemed to be suitable for subsequent transfer of the process to the reactor scale. Although *C. curvatus* exhibited better production properties than *C. laurentii*, the latter was selected since a scant amount of information is currently available regarding its use for biodiesel production. A typical fermentation of *C. laurentii* in RCW medium in shaken flask is reported in Figure S1 and shows that maximal lipid accumulation took place after 24 h from the depletion of N from the growth medium. Taking into account the lipid production peak (5.06 g L^{-1}) and the relative biodiesel yield (27.1%), the volumetric production of FAME derived from lipid transesterification was estimated to amount to 1.37 g L^{-1} (Figure 1B).

3.2 Reactor experiments with C. laurentii

To assess the feasibility of upscaling, the production process with *C. laurentii* was performed in a stirred tank reactor, as shown in Fig. 2.

In STR, nitrogen starvation occurred after only 36 h, thus resulting in an anticipated peak of lipid production (9.93 g L^{-1} at 60 h). The lipid production peak coincided with the occurrence of maximal biomass production (14.37 g L^{-1}). The decrease in total sugars concentration proceeded at an almost linear rate in the early 48 h to dramatically decline thereafter (Fig. 2A). As a consequence, the oxygen percent saturation, after a significant drop in the same time interval, tended to rise again to reach values which approached initial ones. Conversely, the pH did not significantly change throughout the process as shown in Fig. 2B. Noteworthy, at the lipid production peak, the soluble COD was reduced by 83.4% reaching values as low as 3600 mg L^{-1} (data not shown). Table 2 shows that at the time of maximal lipid accumulation, biodiesel yield amounted to 32.64±3.24%; on this basis, the maximal volumetric amounts of total FAME derived from transesterification were estimated to be 3.24 g L^{-1}. As a result of this calculation, $Y_{P/X}$ and $Y_{P/S}$ were 0.23±0.01 and 0.17±0.01, respectively.

Table 2, reporting also the percent FAME composition of *C. laurentii* lipids obtained in STR, shows that the main fatty acids were oleic acid, linoleic and palmitic acids (47.2, 23.7 and 18.5%, respectively). Noteworthy, a significant change in FAME composition was observed in bioreactor as compared to shaken cultures, with a significant decrease in total saturated fatty acids (27.9 vs. 38.2%) and a 2.8-fold increase in linoleic acid (23.7 vs. 8.3%).

4. Discussion

The objective of this study was to investigate the feasibility of a second-generation biodiesel production by oleaginous yeasts grown on a dairy wastewater byproduct (i.e., RCW), for which, as opposed to cheese whey, there is a limited amount of information as a feedstock (Pirozzi et al., 2013; Castanha et al., 2014). Although both initial C/N ratio and absolute N amounts in the RCW-

based medium might be putatively conducive to substantial yeast growth (Beopoulos et al., 2011), only 4 out the 18 strains under study were able to produce a biomass higher than 3 g L^{-1}. The strains that met this requirement were *R. glutinis* UCD 68-255, *T. fermentans* NRRL Y-1492 and two species belonging to the genus *Cryptococcus* (i.e., *C. curvatus* and *C. laurentii*). The *Cryptococcus* and *Trichosporon* genera encompass several species with lactose-assimilating ability. Thus, the failure of the remaining tested strains to substantially grow on RCW might be due to their weak or absent lactose-assimilating ability. In this respect, it may be noted here that lactose-assimilating capacity in yeasts is not very widespread (Frengova et al., 2004) and, for some species, such as *L. starkeii* and *Kluyveromyces marxianus*, this feature has been shown to be strain-dependent (Slodki and Wickerham, 1966; Naumov, 2006). However, irrespective of their growth abilities on RCW, the large majority of the strains under study met the requirement of oleaginicity accumulating intracellular lipids to larger than 20% of their cellular dry mass (Ratledge, 2002) with the only exceptions of *P. membranifaciens* 6C1, *C. rugosa* NRRL Y-95 and *R. glutinis* UCD 68-225.

C. curvatus NRLL Y-1511 was the best performing strain in the screening on the RCW-based medium in terms of production, productivity and lipid accumulation capacity. However, this species is widely recognized as a good lipid producer and this attitude has been already tested on several low-cost substrates, including biodiesel-derived glycerol (Thiru et al., 2011; Tchakouteu et al., 2015), beet molasses (Takakuwa and Saito, 2010), olive mill wastewater (Yousuf et al., 2010), hydrolysates of sweet sorghum bagasse and wheat straw (Liang et al., 2012; Yu et al., 2014) and cheese whey (Seo et al., 2014). On the last substrate, which is highly related to that used in the present study, in particular, a maximum lipid productivity of 4.68 g L^{-1} d^{-1} was obtained. Conversely, the lipid-producing capacity of *C. laurentii* has not been investigated on whey-based media with a sole exception where another strain of this species was tested on cheese whey supplemented with sugarcane molasses (Castanha et al., 2014). In addition, this species deserves

particular attention due to its remarkable ability to use a variety of carbon sources and to its tolerance to potential inhibitors of yeast growth (Sitepu et al., 2014). In the present study, lipid volumetric production and productivity of *C. laurentii* (5.06 g L^{-1} and 1.7 g L^{-1} d^{-1}) were significantly higher than those reported (2.96 g L^{-1} and 0.102 g L^{-1} d^{-1}) in the aforementioned study of Castanha and collaborators (2014) and these parameters were further improved at the reactor scale.

In fact, the process transfer from shaken flask to the STR was successful leading to remarkably improved biomass and lipid productions; on a volumetric basis, the amounts of FAME derived from STR were found to be 2.4-fold higher than in shaken cultures. Moreover, the attainment of the lipid peak was anticipated with respect to shaken flask thus resulting in higher productivity. This anticipation observed in the STR was due to a better mass transfer of both O_2 and nutrients thus leading to an earlier occurrence of nitrogen starvation in bioreactor, an event known to trigger lipid accumulation in oleaginous yeasts. In particular, when nitrogen depletion takes place, the residual carbon in the medium is readily converted to storage lipids (Ratledge, 2002; Papanikolau and Aggelis, 2011). Noteworthy, the productivity observed in the present study was higher than that reported for *C. laurentii* DMKU-AmC14 on a glycerol-based medium (Polburee et al., 2015). Although a marked reduction in organic load was observed in the spent medium in concomitance with the lipid production peak, residual COD values were well above the regulatory standards for effluent discharge into receiving water bodies. However, treatment costs of this spent medium in a wastewater treatment plant would be significantly lower than those for RCW since, in the absence of other critical parameters (e.g., toxic pollutants, chromophoric substances etc.), they mostly depend on residual COD rather than on the volumes conferred.

The determination of the lipid profile of *C. laurentii* indicated the predominance of 16- and 18-carbon chain saturated and monosaturated fatty acids in agreement with Castanha et al. (2014) and this was associated with a low content in PUFA. Noteworthy, the FAME profile of lipids

obtained in the bioreactor significantly differed from that obtained in shaken flask with a concomitant increase in linoleic acid and decrease in total saturated fatty acids. In this respect, dissolved oxygen levels in the medium can markedly modify the fatty acid profile produced by oleaginous microorganisms (Laoteng et al., 2011) and a decrease in unsaturated fatty acids concomitant to the reduction of available oxygen was observed in *Saccharomyces cerevisiae* (Bardi et al., 1999) and *Apiotrichum curvatum* (Davies et al., 1990) cultures. This is not surprising since oxygen acts as the terminal electron acceptor in reactions catalyzed by fatty acid desaturases which play a key role in the synthesis of MUFA and PUFA (Lee et al., 2016); moreover, an oxygen-induced increase in the transcripts levels of desaturase genes was observed in *Mucor rouxii* cultures (Ruenwai et al., 2010). Although an increase in PUFA has been shown to be detrimental to the oxidative stability of the biodiesel (Jakeria et al., 2014), in the present study, the reactor-induced change led to a FAME composition which substantially resembled that of the Jatropha oil (Thiru et al., 2011), the use of which is well established in biodiesel production. It has been reported that oil sources containing high amounts of oleic acid and long chain saturated fatty acids would be ideal candidates for biodiesel purposes, because this composition positively impacts on biodiesel performance parameters, such as cetane number, kinematic viscosity, melting point, oxidative stability and heat of combustion (Knothe, 2005). In this respect, the FAME composition of *C. laurentii* appears to meet these requirements.

4. Conclusions

On the one hand, the screening performed with a variety of strains belonging to well known lipid-accumulating species confirmed that whey-related substrates, such as RCW, are often not adequate to support yeast growth since the large majority of carbon in this byproduct is found as lactose, the assimilation of which is not widespread among yeasts. Thus, a profitable use of this dairy byproduct as a feedstock for microbial production processes involving yeasts would require its

364 pretreatment either by acid hydrolysis and subsequent pH correction or enzymatic hydrolysis with
365 β-galactosidase which is commercially available at low costs. This might be beneficial to lipid
366 production processes involving *L. starkeii* and *R. toruloides*, which despite a limited growth on
367 RCW, maintained a significant lipid accumulation capacity and yielded FAME profiles which
368 were compatible with good biodiesel properties; studies are underway to assess this hypothesis. On
369 the other hand, the successful transfer of the lipid production process of *C. laurentii* grown on
370 RCW from shaken flask to STR offers wide and promising perspectives of further improvements.
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Figure legends

Figure 1. Percent compositions of fatty methyl esters (FAMEs) derived from lipids produced by *C. curvatus* NRRL Y-1511, *C. laurentii* UCD 68-201, *L. starkeii* NRRL 11557 and *R. toruloides* Y-17902 grown in shaken flask on the RCW-based medium (A) and biodiesel yields (%) and FAME amounts obtained from lipid transesterification and referred to unit volume of culture broth (B). Data are referred to the time of maximal lipid accumulation and are the means of six chromatographic runs (2 technical replicates for each independent culture carried out in triplicate). Abbreviations: 16:1, palmitoleic acid; 16:0, palmitic acid; 18:2, linoleic acid; 18:1 oleic acid; 18:0, stearic acid; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Figure 2. Time courses of lipid production and residual nitrogen and total sugar concentrations (Plot A) and biomass production, pH and dissolved oxygen (Plot B) in *C. laurentii* UCD 68-201 cultures grown in a 3-L stirred tank reactor (impeller speed, 600 rpm; aeration rate, 1.5 vvm). Data are the means ± standard deviations of duplicate reactor experiments. Inset in plot A contains a micrograph of 72-h-old cells stained according to Ravikumar et al. (2012) to detect the presence of storage lipid bodies.

Figure S1. Time courses of lipid and biomass productions and concentrations of residual nitrogen and total sugars in *C. laurentii* UCD 68-201 cultures grown in shaken flask. Data are the means ± standard deviations of triplicate cultures.

525 **Table 1.** Biomass (X) and lipid (P) productions, yield parameters ($Y_{X/S}$, $Y_{P/X}$, $Y_{P/S}$) and time of maximal
526 lipid accumulation (t) obtained for each of the 18 yeast strains grown on RCW-based medium.

	X (g L^{-1})	P (g L^{-1})	$Y_{X/S}$	$Y_{P/X}$§	$Y_{P/S}$§	t (h)
C. rugosa NRRL Y-95	1.74±0.01	0.30±0.01	0.13±0.01	0.17±0.01	0.02±0.00	72
C. albidus UCD 68-150	2.00±0.07	0.72±0.10	0.15±0.01	0.36±0.06	0.06±0.01	72
C. albidus UCD 68-174	0.71±0.13	0.35±0.02	0.06±0.01	0.49±0.04	0.03±0.00	48
C. curvatus NRRL Y-1511	10.77±0.21	6.83±0.14	0.50±0.04	0.63±0.02	0.37±0.02	72
C. laurentii UCD 68-201	7.28±0.10	5.06±0.28	0.52±0.02	0.70±0.05	0.36±0.03	72
L. starkeii NRRL 11557	0.79±0.10	0.50±0.07	0.08±0.01	0.63±0.14	0.05±0.01	96
P. anomala AN/4	1.50±0.06	0.34±0.01	0.10±0.01	0.23±0.01	0.02±0.00	72
P. guilliermondii 1067	0.74±0.00	0.35±0.02	0.05±0.00	0.48±0.03	0.02±0.00	72
P. membranifaciens 6C1	2.71±0.20	0.34±0.05	0.31±0.03	0.13±0.03	0.04±0.01	72
R. glutinis DBVPG 3853	0.95±0.05	0.31±0.04	0.07±0.01	0.32±0.06	0.02±0.00	72
R. glutinis UCD 68-255	3.03±0.15	0.27±0.01	0.21±0.01	0.09±0.01	0.02±0.00	72
R. minuta UCD 68-280	0.66±0.03	0.37±0.03	0.06±0.00	0.56±0.07	0.03±0.00	72
R. toruloides NRRL 1091	0.86±0.07	0.41±0.03	0.06±0.01	0.48±0.07	0.03±0.00	72
R. torouloides NRRL Y-17902	0.64±0.03	0.51±0.01	0.05±0.00	0.79±0.05	0.04±0.00	96
T. fermentans NRRL Y-1492	3.50±0.22	0.69±0.06	0.32±0.03	0.20±0.03	0.06±0.01	48
Y. lipolytica NRRL YB-423	1.15±0.19	0.38±0.04	0.08±0.01	0.33±0.09	0.03±0.00	72
Y. lipolytica NRRL Y-1095	1.56±0.08	0.39±0.02	0.18±0.01	0.25±0.03	0.05±0.00	72
Y. lipolytica NRRL Y-7208	1.35±0.15	0.38±0.01	0.18±0.02	0.28±0.02	0.05±0.01	72

527 $Y_{X/S}$=biomass yield; $Y_{P/X}$= specific lipid yield; $Y_{P/S}$= lipid yield referred to consumed sugars; §
528 Calculated by relating total lipids, determined according to Izard and Limberger (2003), to the
529 amounts of total sugars consumed.
530

Table 2. Performance indicators of lipid production process in STR by *C. laurentii* including yields (Biodiesel yield, $Y_{P/S}$, $Y_{P/X}$, $Y_{X/S}$) and biodiesel and biomass production rates (r_P and r_X, respectively), N and total sugars consumption rates (r_N, r_S) specific growth rate (μ) and percent fatty acid composition. All values have been calculated at the time of maximal lipid production.

Parameter	Dimension unit	Value
Yields §		
Biodiesel yield	(%)	32.64±3.24
$Y_{P/X}$ǂ	(g g^{-1})	0.23±0.01
$Y_{P/S}$ǂ	(g g^{-1})	0.17±0.01
$Y_{X/S}$	(g g^{-1})	0.65±0.02
Rates §		
r_Pǂ	(g L^{-1} d^{-1})	1.30±0.09
r_S	(g L^{-1} h^{-1})	0.31±0.00
r_N	(mg L^{-1} h^{-1})	2.49±0.00
r_X	(g L^{-1} h^{-1})	0.24±0.00
μ	(h^{-1})	0.02±0.00
Lipid profile †‡		
Palmitoleic acid	(%)	0.39±0.05
Palmitic acid	(%)	18.53±1.24
Linoleic acid	(%)	23.47±1.01
Oleic acid	(%)	47.16±0.97
Stearic acid	(%)	5.45±0.28
Other Saturated fatty acids (SFA)	(%)	3.93±0.73
Other Monounsaturated fatty acids (MUFA)	(%)	0.30±0.07
Other polyunsaturated fatty acids (PUFA)	(%)	0.77±0.14
Total SFA	(%)	27.91±2.25
Total MUFA	(%)	47.85±1.09
Total PUFA	(%)	24.24±1.15

§Data are the means ± standard deviations of duplicate reactor experiments; ǂCalculated on the basis of the biodiesel yield; †Data are the means ± standard deviations of 4 chromatographic runs (2 technical replicates for each reactor experiment); ‡Predominant fatty acids are listed as a function of increasing retention time.